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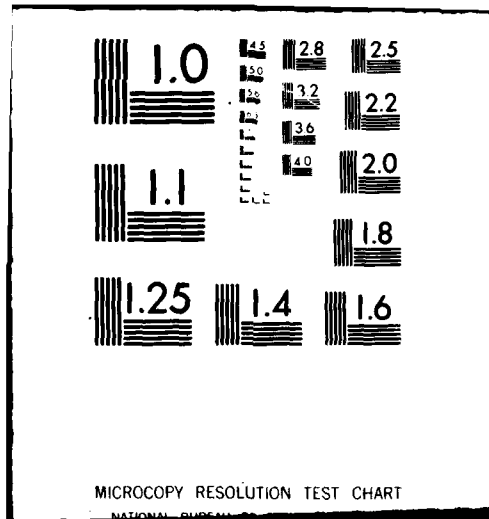
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) This report is a summary of results obtained by our studies of the internal viscosity of red blood cells and spinach thylakoids as observed by electron paramagnetic resonance techniques. Specific detail considers the individual efforts on each of these cell systems, the difficulties in making these measurements, and conclusions based on the results in light of the difficulties. In addition, a discussion on the measurement of the rotational motion of spin labels in phospholipid vesicles is presented as well as a dis-		

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cussion on the applicability of measuring translational diffusion by measuring spin label collision frequency.

The conclusion of the report is that although there are problems in interpretation of the measurements, internal viscosity of cells can be measured reliably by the spin label method.

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"CONTRIBUTIONS OF MEMBRANE COMPONENTS
TO INTRACELLULAR WATER ORDER:
RESEARCH SUMMARY"

by

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PROJECT OBJECTIVES

1. How far does ordered water extend into the cell?
2. What are the contributions of the lipid & protein constituents of the membrane to water order?
3. Does the water inside a cell represent a different state of water compared to bulk water?

PROCEDURES

Procedures are described in detail in Annual Report #2 of this review (See pages 12 through 21) for the preparation of red blood cell ghosts, spinach thylakoids and for the use of potassium ferricyanide and TEMPAMINE (2,2,6,6-tetramethylpiperidine N-oxyl-4 amine) for measuring internal viscosity.

PROJECT EXPERIMENTAL SEQUENCE

Measurement of rotational motion of spin labels

The techniques and mathematical assumptions in determining spin label rotation from relaxation times has been discussed previously (See Annual Report #2). The major limitation of this method is that the spin label must tumble isotropically. A further limitation is that rotational motion may not provide information about the movement of materials within a cell. Spin labels will show rapid rotational motion in solidified epoxy (Keith, private communication). However, it is first necessary to understand the factors limiting rotational motion in cells before one undertakes a study of translational (lateral) motion. This section presents the results we have obtained from measuring spin label rotation in red cells and spinach thylakoids.

Spinach thylakoids

In order to more fully understand the extent to which water restricts spin label rotational motion in a cell, we studied cells which have varying internal aqueous space. Spinach thylakoids have a small internal volume (at high osmolarities and in the dark, their membrane surfaces are only 100 Å apart [Murakami and Packer, 1970]). This seemed the ideal system for studying close range membrane surface effects.

Our initial studies of the motion of TEMPAMINE in the thylakoid aqueous interior showed that TEMPAMINE showed no tendency to bind the thylakoid membranes (although there was some concentration of TEMPAMINE by the intact thylakoids in the dark) and that its motion inside the thylakoids was equivalent to that of TEMPAMINE in aqueous glycerol with a bulk viscosity of 10 cP. Samples prepared by distruption of the thylakoids by 20% Triton X-100 and sonication hindered TEMPAMINE rotation by only a factor of 1.9. Ferricyanide leaked slowly into the thylakoid interior, but this did not affect our measurement of rotational motion. We conclude that in the resting thylakoid, the motion of TEMPAMINE was restricted by a factor of 10 compared to bulk water. This was an interesting finding since the possibility of ice-like water near the membrane inner surface could assist in proton translocation from the site of water splitting and plastoquinone oxidation to the site of ATP synthesis, a relatively long distance away (Berg et al., 1979). Similar experiments in active thylakoids (in the light) are more complex because of the rapid loss of TEMPAMINE signal (probably due to spin label reduction) and the partitioning of TEMPAMINE across the membrane in response to the developing pH gradient (Crofts, 1967).

Additional information about the relationship between thylakoid size and TEMPAMINE motion has been obtained by Berg and Nesbitt (private communications). As thylakoid swell, the rotational motion of TEMPAMINE decreases. This can be explained either by a reduced concentration of intrathylakoid material or by the increased distance between the membrane surfaces. Since there is no known intrathylakoid material other than water, the latter hypothesis seems most probable.

DIFFICULTIES

Because spinach thylakoids have such a small internal aqueous space, even packed cells have a total internal volume of at most 5% of the total sample. Thus the partitioning of the spin label, even a charged entity such as TEMPAMINE, into the membrane becomes a major contributor to the total signal. Sophisticated computer manipulation techniques can resolve a signal which apparently arises from the internal aqueous compartment. Measurement of the hyperfine coupling constant (A_n) gives an indication of the polarity of the spin label's environment (Jost and Griffith, 1976). Our results yield A_n values very close to water (16.0 gauss vs 16.5 gauss for H_2O), but the A_n of TEMPAMINE in oleic acid gives A_n of about 15.5-15.8 gauss (Morse, unpublished observations). Thus, the determination of the physical location of the label in the cell was tenuous. In swollen thylakoids, the assignment of spin label environment is much better (Berg and Nesbitt, private communications), and the motion of TEMPAMINE is still seen to be 3-5x slower than in bulk water.

CONCLUSIONS

We conclude from our studies that TEMPAMINE samples the internal aqueous compartment of spinach thylakoids and that this motion is restricted 3-10x that of bulk water. Some, but not all, of this effect may be explainable by TEMPAMINE present in the membrane. As the thylakoid swells, TEMPAMINE rotational motion increases. This may be due to a combined effect of increasing the internal aqueous signal and diminishing the possible membrane signals as well as reducing the % of internal water in direct contact with the membrane surface (and thus "more" ordered [Berg et al., 1979]).

RED BLOOD CELLS

Previous work (Morse, 1977) had shown that the spin label TEMPAMINE tumbled about 5 times more slowly in the intracellular space of the red blood cell than in bulk water. Thus red cell internal viscosity was measurable by the EPR method. Since the red cell has none of the problems associated with the thylakoid and has a reasonably well defined structure, it seemed a more acceptable and practical system to study. Also, the internal viscosity was postulated to play a role in determination of the viscoelastic properties of the red cell in its movement through the vascular system and particularly the capillaries (Dinfenfass, 1967, 1968).

Experimental

Our studies (Morse et al., 1979,) were directed at determining how the red cell membrane affected spin label motion inside the cell. Control experiments showed that the spin label TEMPAMINE did not bind to hemoglobin or the red cell membrane itself and was sampling the aqueous interior of the cell. We found that as the red cell swells TEMPAMINE rotation decreases. The motion of TEMPAMINE was also not affected by temperature (if spin label motion in bulk water at that same temperature was used as a reference). Increasing pH from 6 to 9 also caused an increase in spin label motion.

This was interpreted in terms of cell swelling with pH changes (Gary-Bobo and Solomon, 1968) but could also be an indication of charge or ionic redistributions within the interior (Salhaney, private communication).

When hemoglobin-free resealed ghosts were prepared (Morse *et al.*, 1979), we observed similar results. However, the motion of TEMPAMINE in the ghost was about a factor of 2.5 more rapid than in intact red cells. This suggested that the hemoglobin clearly played a role in hindering TEMPAMINE motion in the intact cell, but that additional contributions came from the membrane. Indeed, swelling of the ghost caused a decrease in spin label rotation. This must be interpreted as a membrane effect since there is no additional intracellular constituents in ghosts. Hemoglobin alone at concentrations found in intact, isoosmotic-cells (5 mM) hindered TEMPAMINE motion about a factor of 3. Addition of this rotational hinderance with that of the membrane (ghost), gives the factor by which TEMPAMINE motion is hindered in the intact cell. This may be circumstantial, but the result is certainly provocative.

DIFFICULTIES

Measurement of rotational motion in intact red cells is straight forward. Because TEMPAMINE does not bind to hemoglobin and hemoglobin in the oxidized form does not contribute to line broadening processes (shorter relaxation time), then rotational motion is a good measure of the actual rotational diffusion constant. Also, the membrane does not contribute significantly in volume compared to the internal aqueous space. As opposed to thylakoids, A_n in red cells is identical to that in water.

The major difficulty in measuring spin label motion in ghosts is that the ghosts are often very leaky to ferricyanide. Although this doesn't affect the measurement of spin label rotation as such, it requires rather complicated compensation techniques to determine the original line heights (Morse, *et al.*, 1979).

CONCLUSIONS

We conclude that spin labels tumble more slowly in red cells and ghosts than in bulk water and that this is not due to hemoglobin alone. Ghosts which show size changes also show changes in TEMPAMINE rotation. Thus, a membrane surface effect plays a role in this cell as well. Whether it is due to the well-known spectrin-actin internal protein meshwork is conjectural at this point in time.

ROTATIONAL MOTION OF TEMPAMINE IN PHOSPHOLIPID VESICLES

The results obtained from the study of spin label motion in red cell and thylakoids could arise from three considerations. The first is simply that water trapped by a lipid bilayer (membrane) is constrained in some way so that it appears to have a higher viscosity than would bulk water. The second is that the protein and/or lipid charges on the membrane inner surface order water over long distances (longer than a few hundred Angstroms). The third is that membrane proteins themselves affect water order over long distances (non-membrane proteins such as hemoglobin or bovine serumalbumin, have no such effect).

The first consideration can be dealt with by forming lipid vesicles and measuring spin label motion within them. Several methods are available for vesicle formation: the method of Huang (1969) makes vesicles 250-300 Å in diameter (and would thus model thylakoids) while other methods (Deamer *et al.*, 1976), (Olson *et al.*, 1979) form vesicles on the order of 0.2 - 3 microns in diameter. These systems can be used to

study the effect of vesicle size and hence trapped water volume on spin label motion. If a phospholipid is chosen which is uncharged (phosphatidyl choline), then surface effects are minimized.

RESULTS

Several months were spent studying TEMPAMINE motion in the sonicated vesicles. Our studies indicated that TEMPAMINE motion decreased above the phase transition of the lipid used to make the vesicles. Also, the motion of the label below the phase transition was very slow. Although the hyperfine coupling of TEMPAMINE in the vesicles was close to bulk water (16.3 gauss), we were not certain that we were indeed looking only at TEMPAMINE motion in the internal aqueous compartment. We therefore compared the vesicle spectra with those taken of TEMPAMINE in oleic acid. We chose oleic acid as the most polar lipid available in a membrane hydrocarbon region. The spectra from oleic acid showed an A_n value of 16.0 gauss, but this was sufficiently similar to make our previous data suspect. Permanently charged spin labels such as trimethyl TEMPAMINE could not be loaded into the vesicles at sufficient concentration to observe a non-collision broadened spectrum suitable for measuring rotational correlation time.

The synthesis of "Deamersomes" (Deamer *et al.*, 1976, 0.3 microns in diameter) is quite simple and reliable, but only low concentrations of lipids can be used (necessary to prevent vesicle fusion) and thus signal to noise becomes a problem. We surmounted this by generating a pH gradient across the vesicles and using this to concentrate TEMPAMINE inside. In this way we could get an ESR signal. Early results from this preparation show TEMPAMINE motion similar to bulk water.

Preliminary results have been obtained for the motion of TEMPAMINE in dipalmitoyl lecithin vesicles using potassium trioxalatochromate as the broadening agent. TEMPAMINE motion is temperature independent up to 20° C and gives a rotational correlation time equivalent to glycerol-water with a viscosity of 2 cp. However, above the 41° C phase transition, TEMPAMINE motion decreases (equivalent to bulk viscosity of 4). This surprising result is evidence that spin label motion in the interior of lipid vesicles is regulated by membrane fluidity. Further experiments have been done with totally impermeable spin labels (such as trimethyl TEMPAMINE) to eliminate any possibility of membrane-soluble spin labels. Our results with trimethyl TEMPAMINE are similar and strengthen our conclusion. We can rule out the possibility of membrane solubility of the spin label by noting that the polarity-dependent hyperfine coupling constant is equivalent to that of bulk water. This finding, while preliminary, is extremely important in furthering our understanding of how membranes regulate intracellular viscosity.

Difficulties

As with thylakoids, the trapped aqueous volume of sonicated (Huang, 1969) vesicles of 250-300 Å diameter is on the same order as the volume occupied by the lipid. Thus partitioning of the label into the lipid contributes significantly to the signal. All my attempts to measure spin label motion using the spin label TEMPAMINE (pK 9.6) even at low pH, result in serious partitioning problems which presently seen soluble only with enormous computer effort (several hours/spectrum). Results described above are based on 2-3 analyses of this type and are clearly not supported by a statistical study (10-20 runs of 10 spectra each). A possible way around this problem is to trap an impermeable spin label (CAT₁; trimethyl TEMPAMINE) during the sonication process and remove the exogenous label. The above results suggest this is possible, but again a concentration problem arises of a different sort (how many labels in a vesicle represent an average).

The vesicle preps which produce larger vesicles have been more successful. TEMPAMINE is the spin label of choice [attempts to capture trimethyl TEMPAMINE in vesicles made by the method of Deamer et al. (1976) result in severe signal to noise problems due to low capture ratios of the label which are necessitated by the small amount of lipid used]. The method of Olson et al. (1979) is the best so far and has been used to determine the changes in TEMPAMINE motion in vesicles .2 micron in diameter above and below the lipid phase transition (see above). Removal of solvent by extensive evacuation is necessary (Madden and Morse, unpublished observations).

REMAINING EXPERIMENTS

While the results with lipid vesicles indicate that trapping water in a vesicle does not greatly hinder spin label rotational motion, there is much remaining work to be done here. The second and third considerations given above have not been attempted yet. Thus, we do not know the effect of membrane surface change or protein distribution on intracellular spin label motion. These are crucial questions and must be answered before we can understand spin label rotation in cells.

TRANSLATIONAL MOTION

Translational motion of spin labels is derived from considerations of spin label collision frequency (Kivelson, 1960; Plachy and Kivelson, 1967; Eastman et al., 1970; Salikhov et al., 1971; Devaux et al., 1973; Keith et al., 1977). Spin label collisions can result in electron orbital overlap so that the electron can exchange between adjacent nuclei. Likewise, a contribution arises from the presence of the additional nuclear and electronic magnetic dipoles. This latter contribution is less important at low viscosities (high collision frequencies). It has been estimated to contribute about 20% of the line width arising from spin label concentration-dependent line broadening in uncharged spin labels at low viscosities (Dix et al., 1979). The ultimate result of an increase in collision frequency is an increase in line width. However, since many components contribute to total line width, it is necessary to remove non-translational components such as interaction with ^1H , ^{15}N , ^{13}C , dissolved oxygen, and transition metals. In addition, the rotational contribution to line width must be considered as well (Morse et al., 1975).

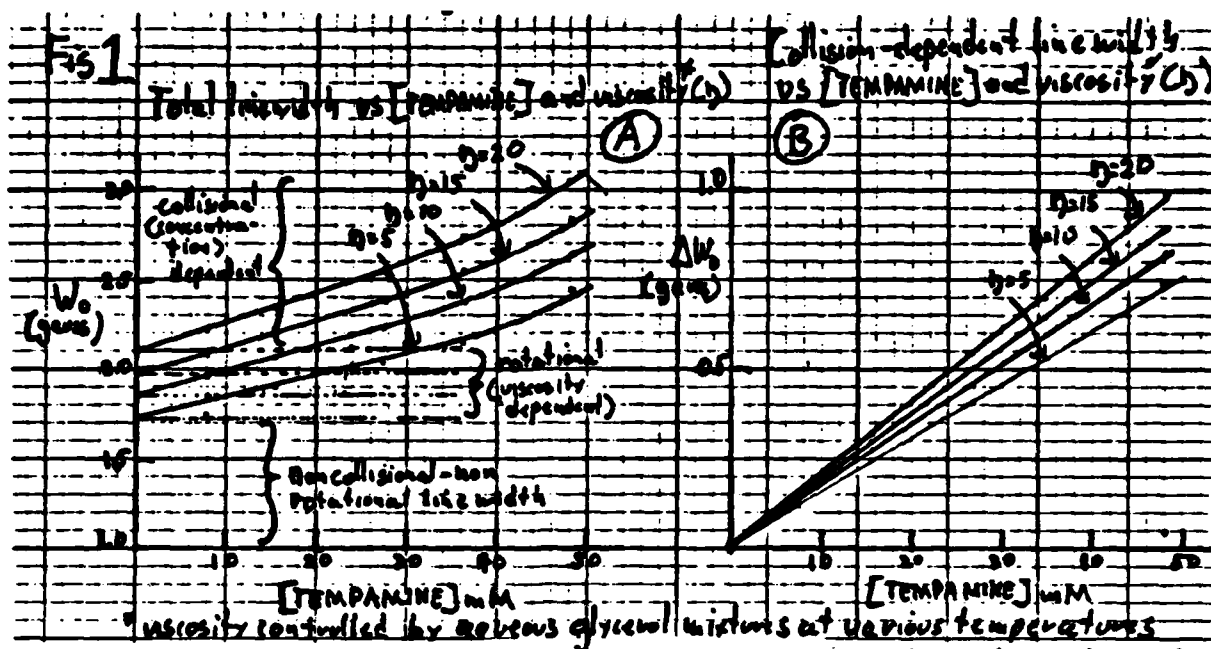
Separation of the collision-dependent spectral line widths from rotational and other contributions can be done experimentally. In the following analysis, we studied line width of TEMPAMINE, a water-soluble spin label, at different viscosities in 65% aqueous glycerol at pH 7.5 where TEMPAMINE is positively charged. When total line width vs TEMPAMINE is plotted, the rotational, collisional, and residual components of line width can be resolved (Fig 1A). When this is replotted as collision-dependent line width vs [TEMPAMINE], the data is described by a series of straight lines with increasing slope at higher viscosities (Fig 1B). The increasing slope probably arises from an increase in dipole contributions to the linewidth as well as spin label charge considerations. However, collision dependent linewidth (ΔH) divided by [TEMPAMINE] is independent of concentration (Table I). Thus, in aqueous glycerol, TEMPAMINE behaves as predicted theoretically (Kivelson, 1960; Dix & Kivelson et al., 1978). In this case, line width (ΔH) is an accurate indicator of spin label collision frequency over a wide range of solvent viscosities and spin label concentrations.

TABLE I
[TEMPAMINE]mM

7.4
14.6
26.8
35.8
48.6

ΔH /[TEMPAMINE] gauss/mole/liter

17.57
18.66
16.23
19.76
19.75

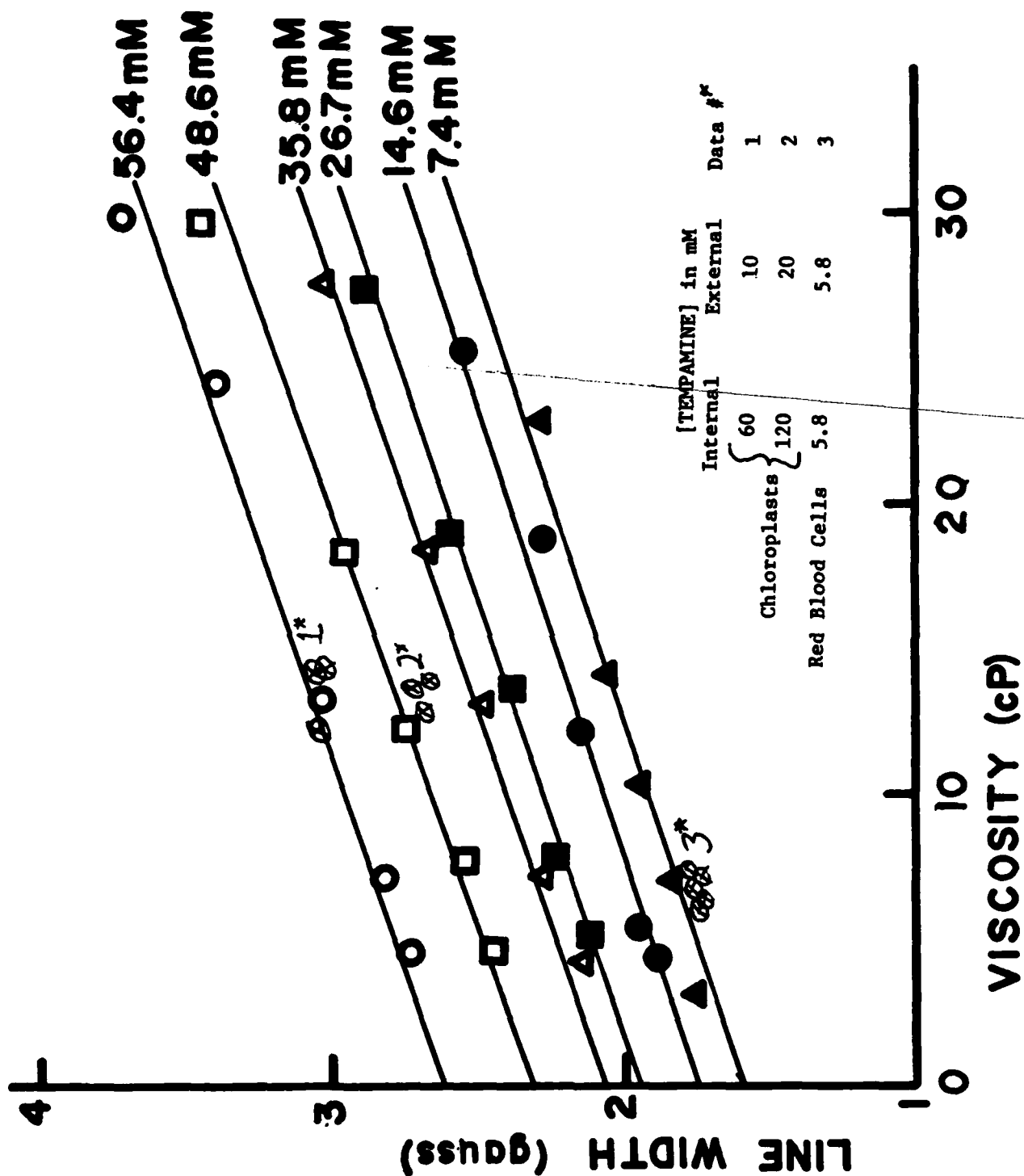


We have performed experiments of the type described above to study the translational motion of TEMPAMINE in red cells and spinach thylakoids. TEMPAMINE is not the best choice because charge repulsion between spin label molecules diminishes frequency of spin label collisions. Nevertheless, we have found that there are differences between spin label translation and rotation.

TRANSLATIONAL MOTION IN SPINACH THYLAKOIDS

Figure 2 shows our results obtained varying spin label concentration and the bulk viscosity of the medium (glycerol-water). These curves are used as reference lines for determining intracellular translational motion. From determinations of integrated intensity of the TEMPAMINE spectra and estimates of the intrathylakoid volume by inulin exclusion (Rottenberg *et al.*, 1972, Ort and Dilley, 1976), it is possible to determine the internal thylakoid concentration of TEMPAMINE at any external TEMPAMINE concentration. When TEMPAMINE is 10 mM outside, it is 60 mM inside; when 20 mM outside, it is 120 mM inside. Based on these concentrations; we can calculate the line widths of 60 and 120 mM TEMPAMINE in glycerol-water with a bulk viscosity of 10 cP (that is the same as the rotational motion of TEMPAMINE inside the thylakoids (Berg *et al.*). When the line widths of intrathylakoidal TEMPAMINE are compared with TEMPAMINE in glycerol-water at the same viscosity and concentrations, we find that the 60 mM data gives a line width approximating a 40 mM TEMPAMINE solution in glycerol-water at 10 cp and the 120 mM data gives a line width approximating a 60 mM solution.

There are several ways to explain this data. One is that translational diffusion is restricted relative to rotational diffusion for all the intrathylakoidal TEMPAMINE. Another is that some of the TEMPAMINE is bound and only the mobile fraction is free to participate in collisions. A third is that there exists diffusion barriers within the intrathylakoid space. The data does show clearly, however, that translational diffusion of spin labels in the thylakoid aqueous space is of the same magnitude as rotational diffusion. This supports my contention that intracellular components are experiencing a motionally restrictive environment.



TRANSLATIONAL MOTION IN RED BLOOD CELLS

Figure 2 also show that the translational motion of TEMPAMINE in red blood cells is essentially equivalent to its rotational motion. TEMPAMINE concentration was also determined within the cells by double integration of the spectra and dividing this number by the hemotocrit to get a mean intracellular TEMPAMINE concentration. When the line widths of 5 mM TEMPAMINE in a solution of glycerol-water of 5 cp was compared to equivalent concentrations and line widths in the intact red cells, the line widths were found to be the same. Thus, translational motion of TEMPAMINE is restricted to the same extent as rotational motion, i.e., by a factor of 5 compared to bulk water. This strengthens the argument that TEMPAMINE motion within red cells is in fact hindered.

FINAL CONCLUSIONS

The conclusion reached by this project are:

- 1) Spin label motion, both translation and rotational, can be studied in a wide variety of cells.
- 2) The results always show that spin label rotational motion in the internal aqueous space of cells is slower than in bulk water and that rotation diffusion is therefore hindered.
- 3) Hindered spin label rotation is not due to spin label binding to cell constituents.
- 4) Translational motion of spin labels in red blood cells is predictable from spin label rotation.
- 5) Translational motion of spin labels in spinach thylakoids is less than expected from their rotational motion.
- 6) Hindered spin label motion is not due solely to trapping water in a vesicle and is regulated by membrane fluidity.
- 7) The partitioning of spin labels into cells and vesicles with a small ratio volume of internal space: membrane volume is a severe and limiting problem.
- 8) The problem can indeed be resolved by the spin label method.

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